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PRINCIPAL INVESTIGATOR: Pauline M. Schwartz, Ph.D.

CONTRACTING ORGANIZATION: VA Connecticut Healthcare System
West Haven, Connecticut 06510

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Pauline M. Schwartz, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES

VA Connecticut Healthcare System
West Haven, Connecticut 06510
E-Mail: pauline.schwartz@yale.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The goal of this grant is to develop a prototype epidermal biosensor for carcinoembryonic antigen (CEA). An epidermal biosensor is a new approach for the early continuous, in vivo detection of the onset of disease by the using genetically modified skin cells to respond to molecules secreted by tumor cells.

The research we have conducted during the first and second year of the grant has allowed us to conclude that human keratinocytes in vitro can be engineered to express a chimeric cell surface receptor and that these modified cells can recognize and bind CEA. We have resolved technical problems arising in the 2nd year. We have proposed a new plan to generate our primary construct and have proposed an alternate chimeric receptor based on the EGF receptor.

We remain confident that we can generate chimeric receptors that can be expressed in keratinocytes that will permit human keratinocytes to recognize and bind CEA. If CEA binding can be made to trigger signal transduction through a chimeric receptor then it should be possible to develop effective epidermal biosensors for early, continuous in vivo detection of breast cancer. Such an early detection system is likely to play an important role in the management of breast cancer espeically in women who have known risk factors.

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INTRODUCTION

The goal of this grant is to determine if it is possible to develop an epidermal biosensor for carcinoembryonic antigen (CEA) as an early, sensitive detector of the onset of breast cancer. An epidermal biosensor represents a new approach to detection of disease whereby a small area of skin containing modified keratinocytes recognizes and responds to molecules secreted into the circulation by a tumor. In the prototype under investigation, epidermal keratinocytes will be engineered to express a chimeric cell surface receptor that will bind CEA and initiate a local reaction. The plan is to design and test the function of chimeric receptors. The long-term objective is to explore the use of epidermal biosensors as a continuous, *in vivo* monitors for the presence of tumor antigens expressed by breast cancer. The expectation is that epidermal biosensors could provide early detection of the onset of disease for high-risk patients so that appropriate medical management could be initiated when it is most likely to result in a positive outcome.

BODY

Statement of Work and rationale for modified chimeric receptor

The overall goal and specific aims focus on the development of an epidermal biosensor for carcinoembryonic antigen (CEA) for the early detection of breast cancer. As noted in the July, 2000 annual report, we modified the key receptor we hoped to genetically engineer that would bind and respond to CEA. Thus, our proposed receptor construct incorporates a single chain variable fragment (scFv) antibody to CEA as the ligand binding domain in chimeric cell surface receptor fused to the transmembrane and intracellular domains of tumor necrosis factor α receptor I (TNFαRI).

The Statement of Work describes the tasks to develop and investigate the modified chimeric receptor for CEA.

Statement of Work and Progress for Years 1 and 2

Progress

Specific Aim 1. Engineer CEA–binding chimeric receptors

Technical Objectives 1. Prepare plasmid constructs

- Task 1. Generate construct #1 -- pRSV scFv anti-CEA Fc γ — **DONE**
- Task 2 Generate construct # 2 -- scFv anti-CEA – TNFα receptor I — **DONE**

Technical Objective 2. Prepare retroviral expression vectors

- Task 1. Generate retroviral vectors for construct #1 — **DONE**
- Task 2. Generate retroviral vectors for construct #2 — **DONE**

Specific Aim 2. Introduce and investigate chimeric CEA-binding receptors in human keratinocytes *in vitro*.

Technical Objective 1. Transduce keratinocytes with and determine chimeric receptor expression

Task 1. Transfect keratinocytes with retroviral vector for construct #1 and select transfected cells resistant to G418. **DONE**

Task 2. Transfect keratinocytes with retroviral vector for construct #2 (CEA – TNFαRI) and select transfected cells resistant to G418. **DONE**

Technical Objective 2. Measure binding of CEA to transduced cells

Task 1. Prepare stock of I-125 CEA **DONE**

Task 2. Measure binding of I-125 CEA to keratinocytes expressing construct #1 **DONE**

Task 3. Measure binding of I-125 CEA to keratinocytes expressing construct #2 CURRENT

Technical Objective 3. Evaluate activity in response to CEA for construct #2.

Task 1. Optimize IL-1 and IL-6 assays for keratinocytes PLANNED

Task 2. Measure IL-1 and IL-6 activity in modified keratinocytes treated with CEA. PLANNED

Specific Aim 3. Examine activity of CEA-TNFαRI receptor in human keratinocytes *in vivo*.

Technical Objective 1. Generate cultures of keratinocytes and transplant onto mice.

Task 1. Prepare composite cultures with keratinocytes expressing functional CEA-TNFαRI receptors. PLANNED

Task 2. Graft composite cultures onto immunosuppressed mice PLANNED

Technical Objective 2. Evaluate response of epidermal biosensor to CEA and CEA-secreting tumors

Task 1. Administer CEA i.v. and compare response to control PLANNED

Task 2. Implant CEA-secreting breast cancer cells and measure time-course and response of epidermal biosensor PLANNED

Rationale for the scFv anti-CEA TNFαRI chimeric receptor in keratinocytes

The objective of this grant is to generate an effective chimeric receptor, i.e. one that would recognize and respond to CEA, and test its capacity to function in human keratinocytes to produce a cellular and tissue response in the presence of CEA. Cell surface receptors have three critical domains: an extracellular ligand binding domain, a transmembrane domain and an intracellular effector domain. Recent literature supports the concept of single chain variable fragment (scFv) antibody as a ligand binding domain in chimeric cell surface receptors [1-5]. A chimeric receptor possessing a scFv antibody the recognizes CEA has been described and a

plasmid containing the construct was obtained from P. Darcy [1].

For the transmembrane and intracellular domains, the structure of TNFα receptor I was chosen. Keratinocytes express a 55kd TNFαR1 that binds TNFα [6] . Keratinocytes respond to TNFα by up-regulation of IL-1 and IL-6 and other cytokines resulting in inflammation [6-8]. TNFαR1 (CD120a) activation requires participation by one or more TNF receptor association factors (TRAF's) which may bring about receptor aggregation and signaling through the NF-kappaB/c-jun pathway [9,10]. Ligand binding is thought to dissociate a factor (SODD – silencer of death domains) from TNFαR1 allowing other factors to associate and produce an active signaling complex. It is hypothesized that CEA binding through an scFv ligand binding domain will activate TNRαRI and trigger its signal transduction pathway.

Tasks accomplished in years 1 and 2

1. Generation of retroviral vector containing the scFv anti-CEA Fc γ cDNA.

The pRSV anti-CEA Fc γ was obtained from P. Darcy [1] . The plasmid was grown and characterized. The plasmid was used for generating a retroviral vector for stable transduction of human keratinocytes [11]. Briefly, the plasmid was transfected into PE501 ecotropic packaging cell line and the resulting virus particles used to infect the PA317 amphotropic cell line. The PE501 and PA317 cell line were obtained from Organogenesis Inc, Canton MA. The medium from the infected PA317 cells containing the infectious, replication-defective retrovirus expressing the cDNA for scFv anti-CEA Fc γ chimeric receptor and the cDNA for neo to confer resistance to G418.

2. Transduction of human keratinocytes in vitro.

Primary human keratinocytes and HaCaT cells were transduced with the retroviral vector expressing the cDNA for the chimeric receptor. Cells expressing the chimeric receptor were selected by growth in medium containing G418.

3. Demonstration of expression of the chimeric receptor.

The cDNA sequence expressed by the retrovirus contained the c-myc tag in the intracellular domain of the chimeric receptor. Cells were stained for the presence of c-myc epitope. Figure 1 shows that the majority of transduced keratinocytes displayed bright fluorescent staining for the c-myc epitope. Untransduced cells showed no staining.

4. Preparation of I-125 CEA.

CEA was purchased from Sigma (St Louis, MO) and labeled with I-125 using the IODO-GEN tubes (Pierce).

5. Demonstration of binding of CEA to CEA-Fcγ keratinocytes.

Keratinocytes were exposed to I-125 CEA. Untransduced keratinocytes retained little radioactivity whereas transduced keratinocytes showed significant binding to I-125 CEA (Figure 2).

6. Construction of plasmid expressing scFv anti-CEA TNFαRI

The scFv anti-CEA sequence was excised from plasmid expressing construct #1 (scFv-CEA-Fc γ) and this segment extended by PCR with primers to allow insertion of the sequence into the Bgl II and Hind III sites of the retroviral expression plasmid pLNCX-2 (Clontech, Palo Alto, CA)

The cDNA expressing TNFαRI has been cloned [12] and a plasmid containing the cDNA was obtained from J. Pober (Yale Univ.). The plasmid was grown and characterized. Since the sequences incorporating the transmembrane and intracellular domains had a Hind III site, a primer was synthesized to allow the TNFαRI domains to be inserted in phase through the NotI and ClaI sites of the scFv-CEA /pLNCX-2 vector. The resulting 1830 base pair construct was transformed into DH5α and plasmid isolated from a large scale preparation.

7. Generation of a retroviral vector for scFv anti-CEA TNFαRI

The pLNCX-2 retroviral expression vector containing the scFv-CEA TNFαRI sequence was used to transfect the RetroPack PT67 packaging cell line (Clontech) using LipofectAmine (GibcoBRL, Rockville, MD). Cells resistant to G418 (500ug/ml) were selected for generation of the defective, infectious retrovirus containing the gene for scFv anti-CEA TNFαRI.

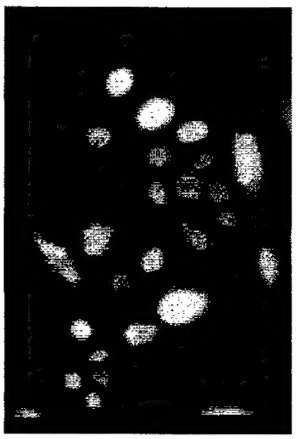
8. Transfection and selection of human keratinocytes and HaCaT cells

Virus from the PT67 cells transfected with the vector containing the scFv anti-CEA TNFαRI was used to infect HaCaT cells in the presence of polybrene [11]. Stable, transfected HaCaT cells were grown in G418 (600ug/ml) and used for further study.

9. Binding of I-125 CEA to CEA-TNFαRI HaCaT cells

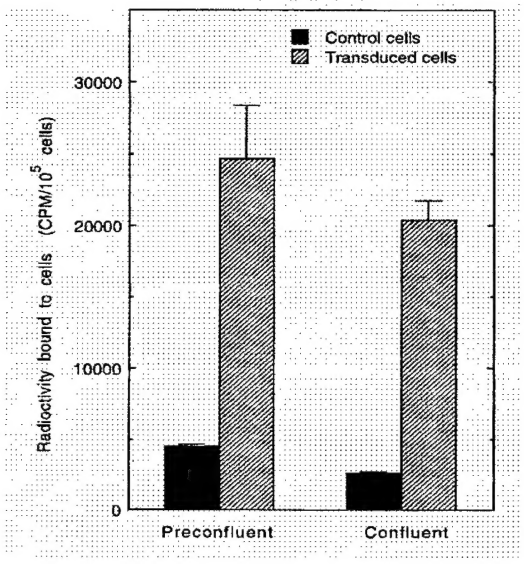
Binding of I-125 CEA was performed as previously carried out for the human keratinocytes expressing construct #1, scFv-CEA Fcγ (Figure 2). Binding of I-125 CEA to scFv-CEA TNFαRI HaCaT cells was compared to binding of I-125 CEA to scFc-CEA Fcγ HaCaT cells (positive control with construct #1). No binding of CEA was observed in the scFv-CEA TNFαRI HaCaT cells but binding was measured to the positive control cells (Figure 3). For further discussion, see next section on problems in accomplishing tasks and plans for 3rd year experiments.

Figure 1. Immunofluorescent localization of scFv-CEA—Fcγ to cell surface of keratinocytes.



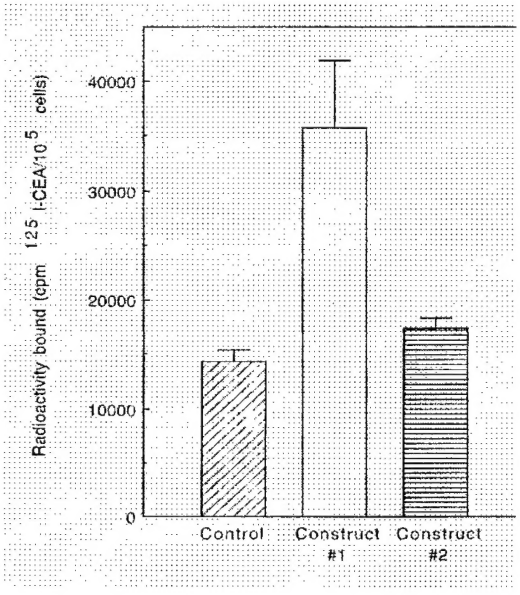
G418-selected keratinocytes transduced with a MMLV retroviral vector expressing the scFv-CEA – Fcγ chimeric receptor were grown on 4-well glass culture slides. After 48hrs. cells were fixed in cold acetone. The presence of the c-myc tag sequence in the extracellular hinge domain of the receptor was detected by staining with anti-c-myc (Sigma) and FITC-anti-mouse IgG (Sigma). Controls, 1° antibody negative and untransduced keratinocytes, had only diffuse background staining (not shown). About 60% of transduced cells displayed bright staining.

Figure 2. Binding of ^{125}I-CEA to Human Keratinocytes Transduced with a Retroviral Vector for Expressing scFv-CEA – Fc-γ.



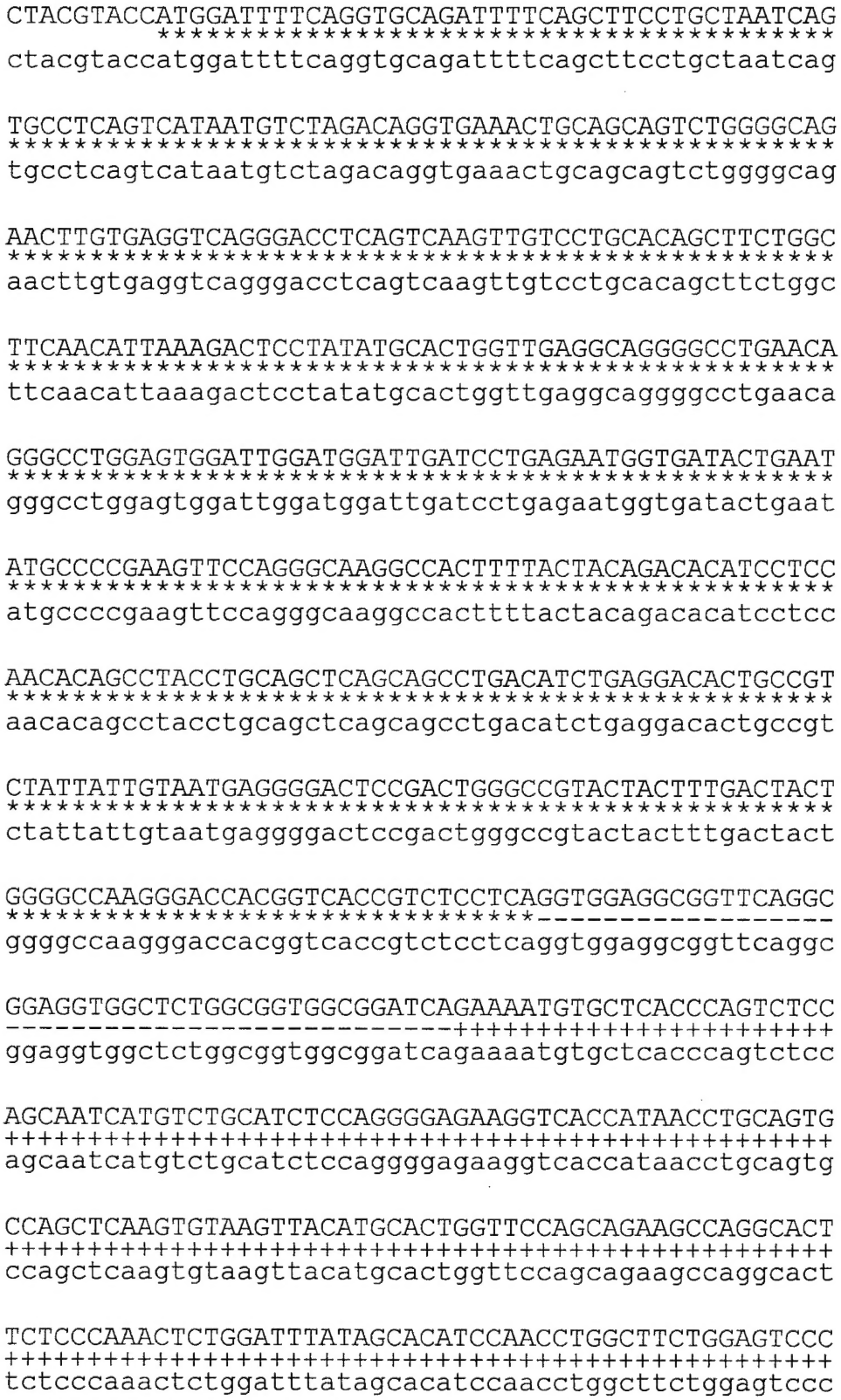
Keratinocytes were seeded in 4-well dishes and allowed to grow for 48hrs. Cells were seeded at different densities to give preconfluent and confluent cultures. Complete MCDB growth medium was removed and replaced with MCDB containing ^{125}I-CEA for 45 mins at 37°C. After removal of the unbound CEA, cell monolayers were washed twice with PBS and precipitated with 10% TCA. The precipitate was re-dissolved in 0.2M NaOH, 0.2% SDS and radioactivity associated with the cells was determined in a gamma counter. Each bar represents the mean ±SD for 3 wells divided by the cell number determined by Coulter counter from 2 wells grown under identical conditions.

Figure 3. Binding of ^{125}I-CEA to HaCaT Cells and HaCaT Cells Transduced with a Retroviral Vector for Expressing scFv-CEA – Fc-γ (Construct #1) or scFv-CEA- TNFαRI (Construct #2).



Cells were seeded in 4-well dishes and allowed to grow for 48hrs. Complete MCDB growth medium was removed and replaced with MCDB containing ^{125}I-CEA for 45 mins at 37°C. After removal of the unbound CEA, cell monolayers were washed twice with PBS and precipitated with 10% TCA. The precipitate was re-dissolved in 0.2M NaOH, 0.2% SDS and radioactivity associated with the cells was determined in a gamma counter. Each bar represents the mean ±SD for 3 wells divided by the cell number determined by Coulter counter from 2 wells grown under identical conditions.

Figure 4. Proposed and Sequenced Construct for scFv-anti-CEA-TNFαR1



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Proposed sequence in UPPERCASE. Actual sequence in lowercase.
Domains: ****** V_H ; ----- Link; +++++ V_L ;^^^^^ c myc tag;<<<<<<< hinge;
. . . . missing sequences; ####### TM;~~~~~~~ IC

Problems in accomplishing tasks and recommended changes for 3rd year experiments

No binding of CEA was observed in the scFv-CEA TNFαRI HaCaT cells but binding was measured to the positive control cells (Figure 3). Western blotting of cells extracts from the scFv-CEA TNFαRI HaCaT cells failed to detect the c myc tag but this procedure was not successful on the positive control cells, scFc-CEA Fcγ HaCaT (data not shown).

We removed the sequence we designed for the scFv-CEA TNFαRI from the retroviral plasmid (from BglII to ClaI) and requested primer walking DNA sequencing of the 1.6kb segment from the W.M. Keck Biotechnology Laboratory at Yale University. The DNA we submitted was out of frame from the proposed sequence because of an interfering restriction site that was not reported on our output (Figure 4).

We propose the following plans for our 3rd year to solve the current problem and learn if we can obtain a chimeric receptor that recognizes and responses to CEA.

Task 1. Re-engineer the scFv-CEA TNFαRI receptor and sequence the construct to insure that it is in frame. Generate the retroviral vector using the correct sequence and introduce it into human keratinocytes and HaCaT cells. Measure I-125 CEA binding to the transfected cells and measure the response of the transduced cells to CEA by determining the activity of IL-1 and IL-6 in control and treated cells.

Task 2. In the event that we do not find binding of CEA or response to CEA from the scFv-CEA TNFαRI transduced cells, we plan to construct a scFv-CEA EGFR construct. The EGF receptor is expressed on human keratinocytes and the cDNA has been cloned [13, 14]. We believe this to be a useful alternate chimeric construct to investigate for receptor activity.

KEY RESEARCH ACCOMPLISHMENTS

In years 1 and 2 of the grant we have learned that:

1. Human keratinocytes in vitro can be engineered to express a chimeric cell surface receptor.

2. Human keratinocytes expressing a chimeric cell surface receptor will recognize and bind a foreign ligand.

REPORTABLE OUTCOMES

Based on the findings generated during the first year of this grant, other applications have been submitted which further explore the development of epidermal biosensors.

Funded application to NCI – Division of Cancer Prevention
STTR Phase I application with Organogenesis Inc.

Publications

NB: Publication and patent applications were submitted prior to award of this grant.

Milstone, L.M. and Schwartz, P.M. Engineering better skin. In: Skin, The Barrier Zone, S. Klaus and J. Hamburger, eds. (in press).

Provisional Patents (Disclosed to Yale University School of Medicine):

Schwartz, P.M. Epidermal Biosensors: Continuous, Early, Sensitive Monitors for Onset of Specific Diseases.

Schwartz, P.M. An Epidermal Biosensor for Carcinoembryonic Antigen.

CONCLUSIONS

The research we have conducted during the first and second year of the grant has allowed us to conclude that human keratinocytes in vitro can be engineered to express a chimeric cell surface receptor and that these modified cells can recognize and bind CEA.

Technical problems arising in the 2nd year have stalled our testing of the scFv-anti-CEA TNFαRI construct. Now that this problem has been identified, a clear plan has been outlined to generate the chimeric receptor. In addition, an alternate chimeric receptor based on the EGF receptor has been proposed.

We remain confident that we can generate chimeric receptors that can be expressed in keratinocytes that will permit human keratinocytes to recognize and bind CEA. If CEA binding can be made to trigger signal transduction through a chimeric receptor then it should be possible to develop effective epidermal biosensors for early, continuous in vivo detection of breast cancer. Such an early detection system is likely to play an important role in the management of breast cancer espeically in women who have known risk factors.

REFERENCES

1. Darcy, P., et al., *Expression in cytotoxic T lymphocytes of a single-chain anti-carcinoembryonic antigen antibody.* Eur J Immunol,, 1998. **28**: p. 1663-72.

2. Fitzer-Attas, C., et al., *Harnessing Syk family tyrosine kinases as signaling domains for chimeric single chain of the variable domain receptors: Optimal design for T cell activation.* J Immunol, 1998. **160**: p. 145.

3. Patel, S., et al., *Impact of chimeric immune receptor extracellular protein domains on T cell function.* Gene Therapy, 1999. **6**: p. 412-419.

4. Wang, G., et al., *A T cell-independent antitumor response in mice with bone marrow cells retrovirally transduced with an antibody/Fc-gamma chain chimeric receptor gene recognizing a human ovarian cancer antigen.* Nature Medicine, 1998. **4**(2): p. 168-72.

5. Hombach, A., et al., *A chimeric receptor that selectively targets membrane-bound carcinoembryonic antigen (mCEA) in the presence of soluble CEA.* Gene Therapy, 1999. **6**(2): p. 300-4.

6. Trefzer, U., et al., *55-kd Tumor necrosis factor receptor is expressed by human keratinocytes and plays a pivotal role in regulation of human keratinocyte ICAM-1 expression.* Journal of Investigative Dermatology, 1991. **97**(5): p. 911-6.

7. Kutsch, C.L., D.A. Norris and W.P. Arend, *Tumor necrosis factor-α induces interleukin-1α and interleukin-1 receptor antagonist production by cultured human keratinocytes.* Journal of Investigative Dermatology, 1993, **101**: p79-85.

8. Aragane, Y., et al., *Transforming growth factor-alpha induces interleukin-6 in the human keratinocyte cell line HaCaT mainly by transcriptional activation.* Journal of Investigative Dermatology, 1996, **106**: p1192-7.

9. Jiang, Y., et al., *Prevention of constitutive TNF receptor 1 signaling by silencer of death domains [published erratum appears in Science 1999 Mar 19;283(5409):1852].* Science, 1999. **283**(5401): p. 543-6.

10. Ledgerwood, E.C., J.S. Pober, and J.R. Bradley, *Recent advances in the molecular basis of TNF signal transduction.* Laboratory Investigation, 1999. **79**(9): p. 1041-50.

11. Garlick, J.A., et al., *Retrovirus-mediated transduction of cultured epidermal keratinocytes.* J Invest Dermatol, 1991. **97**(5): p. 824-9.

12. Gray, P.W., et al., *Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(19): p. 7380-4.

13. Jost, M., C. Kari, U. Rodeck, *The EGF receptor – An essential regulator of multiple epidermal functions.* European Journal of Dermatology, 2000. **10**: p505-510.

14. Xu, Y-h., et al., *Human epidermal growth factor receptor cDNA is homologous to a variety of RNAs overproduced in A431 carcinoma cells.* Nature, 1984. **309**: p 806-10.

APPENDICES

Publication: Milstone, L.M. and Schwartz, P.M. Engineering better skin. In: Skin, The Barrier Zone, S. Klaus and J. Hamburger, eds. (in press).

Curriculum vitae: Pauline M. Schwartz, Ph.D.

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in, Skin:The Barrier Zone
Sidney Klaus and Joseph Hamburger, eds.

ENGINEERING BETTER SKIN

Leonard M. Milstone, M.D. and Pauline M. Schwartz, Ph.D.

Department of Dermatology

Veterans Affairs Medical Center

and

Yale University School of Medicine

P.O. Box 208059

New Haven, CT. 06520-8059

U.S.A.

Mammalian skin is a remarkably effective barrier. As we have heard over the past several days, parasites and insect vectors have developed a variety of physical tools, enzymes and other molecular strategies needed to breach the skin barrier. Parasites also commonly trick the host and evade detection as they pass through the skin using poorly understood stealth mechanisms. Previous speakers have identified the reasons for these adaptations: the skin is richly endowed with rapid and redundant response mechanisms to resist assault and to repair barrier integrity. In light of its clever design and effective performance, it is with some hesitancy and humility that we have considered ways to improve the epidermis.

Moving genes for the purpose of improving nature, otherwise known as genetic engineering, is changing our views of how we treat disease and where we draw the borders between disease and well being. Current technologies can be used to amplify existing traits or to introduce new traits into virtually any living organism; any tissue can be genetically modified, including skin. Yet there is debate as to whether the outcomes of genetic engineering represent biological improvements. This debate is particularly sharp in higher organisms where we neither can reliably predict all the consequences of our efforts nor can we select and clone our best efforts.

In this review, we provide a brief overview of technical issues relevant to genetic engineering in skin. Our own work reflects the bias that the *engineering* part of genetic engineering implies improvement in the efficiency or activity of an existing function, not the creation of inherently new or distinctive forms or functions. Toward that end, our labs have undertaken efforts to use genetic engineering to augment or broaden the skin's normal function as 1) a biosensor of internal health and well-being, 2) an organ of excretion via desquamation, and 3) the target tissue for restoring genes lost during evolution. We will give examples of how

changes in the skin can alert the clinician to the existence of disease; we will show how augmenting iron loss through desquamation might be used to treat the toxic effects of hemochromatosis; we will indicate how vitamin C might be synthesized in the skin.

Delivering genes to the skin: There are two main approaches to delivering genes to the skin (1-3). The *ex vivo* approach usually involves culturing autologous cells, transducing them with high efficiency viral vectors (e.g. retrovirus, adenovirus, adeno-associated virus), selecting cells for appropriate new gene expression and transplanting selected cells back to the host. The *ex vivo* approach is advantageous when control over the level of expression is important and long-lasting expression is an objective. Its main disadvantage is that it is labor intensive, especially when low efficiency, non-viral methods of transduction are used. The *in vivo* approach utilizes direct transfer of genes into skin via injection, using either a syringe or a biolistic particle accelerator ("gene gun"), or via topical application of naked or liposome-encapsulated DNA. The *in vivo* approach is generally less efficient and predictable than the *ex vivo* approach, but may be advantageous when highly localized and transient gene expression are desirable. For applications related to parasitic diseases, the *in vivo* approach may have distinct advantages.

Why choose keratinocytes?: Gene transfer has been accomplished in skin keratinocytes, fibroblasts, endothelial cells and melanocytes. The advantages of keratinocytes for new gene expression in the skin include: ready accessibility; large potential for population expansion *in vitro*; well-established methods for transplantation; high rate of metabolic activity; high density per mm^3; demonstrated ability to secrete peptides into the systemic circulation. For applications related to infectious or parasitic diseases of the skin, keratinocytes would be particularly

attractive targets for genetic manipulation because of their ability to deliver a genetically-encoded toxin or repellant to the outermost limits of the barrier.

Engineering an epidermal biosensor: Skin is a well-recognized, sensitive indicator of internal disease. An engineered, epidermal biosensor would provide an early signal of disease onset, even before traditional signs and symptoms appear. Epidermal biosensors use keratinocytes to detect specific circulating molecules and, by coopting an existing signal transduction pathway, induce a visual or otherwise measurable readout. Epidermal biosensors can be designed to allow continuous monitoring of endogenous molecules (e.g. glucose), or to detect newly appearing molecules associated with a disease (e.g. serum tumor antigen) or an environmental exposure (e.g. toxic chemical). For example, an epidermal biosensor might be designed to detect circulating prostate serum antigen (PSA) in patients at risk for prostate cancer or to detect carcinoembryonic antigen (CEA) in patients at risk for recurrence of bowel cancer. The strategy that we are exploring is to utilize the keratinocytes' ability to initiate an inflammatory response following activation of their cell surface receptors for Fc–γ or Il-1 (4-6). Keratinocytes can be engineered to express chimeric receptors, which utilize the transmembrane/intracellular effector domain of either the native Fc-γ or Il-1 receptors fused to a novel ligand-binding domain. The ligand-binding domain is generated by splicing together the variable domains of heavy and light chains of antibodies known to bind ligands of interest (7). In our work these single chain fragments of the heavy and light chain variable domains (scFv) are chosen to selectively bind circulating tumor antigens such as PSA or CEA. Increasing knowledge about the design and function of chimeric receptors (8,9) and the capacity to develop large libraries of scFv's that bind a wide variety of ligands (7) will be instrumental in developing useful epidermal biosensors. We

are currently testing the hypothesis that CEA interaction with a CEA/Fc-γ chimeric receptor expressed on genetically engineered keratinocytes will initiate intracellular signals that result in a tissue response of erythema or inflammation. Alternatively, it should be possible to design epidermal biosensors for which a readout of oxygen pressure, fluorescence or hyperpigmentation could be measured by a transcutaneous scanning device.

Engineering epidermis for remediation of potentially toxic chemicals: There are two ways in which the epidermis can eliminate potentially toxic materials from the general circulation: by catabolism and return to the circulation of a non-toxic metabolite or by acting as a sink that first traps the toxin intracellularly and then removes it through the normal process of desquamation. Many years ago we calculated that the epidermis had the capacity to degrade large amounts of circulating nucleotides (10). Children with adenosine deaminase deficiency develop combined T and B cell immunodeficiency because of high circulating levels of adenosine. Introduction of the normal adenosine deaminase gene into a small fraction of a patient's deficient keratinocytes should be able to metabolize sufficient circulating adenosine to ameliorate the disease (11). This would represent remediation by epidermal catabolism.

Toxin elimination through desquamation is the second way of engineering keratinocytes for the purpose of dermatoremediation. The epidermis turns over or renews itself every 24-28 days. The keratinocytes that are sloughed from the surface, or desquamated, might be engineered to bind and eliminate or excrete toxins through the process of desquamation. As an example of how this might be accomplished, we have been studying hemochromatosis, a disease of iron overload. Under normal circumstances, absorption of dietary iron is limited by feedback mechanisms in the gut. When too much iron gets into the blood, either through a genetic defect

that leads to unregulated iron absorption or as a result of injections of iron through repeated blood transfusions, iron accumulates and impairs function of critical tissues. Incidentally, the epidermis acts as a biosensor of iron buildup: hyperpigmentation or bronze diabetes is a characteristic, though relatively insensitive cutaneous indicator of hemochromatosis. Under normal circumstances, about 10 percent of absorbed iron is eliminated through desquamation of keratinocytes (12). When more iron is delivered to the skin, as in hemochromatosis, more is eliminated through the skin by desquamation. When skin turns over more rapidly in hyperproliferative diseases such as psoriasis, a greater proportion of absorbed iron is lost through the skin. Clearly the skin has the capacity to accept and eliminate increased amounts of iron. How might we load more iron into the skin?

Iron normally circulates bound to a carrier protein, transferrin. In most tissues, uptake of iron is dependent on a cell surface receptor for transferrin. In epidermis, the transferrin receptor is only expressed on the proliferating basal keratinocytes (13). It is presumed that iron accumulates only in basal keratinocytes; any iron in a suprabasal keratinocyte got there before the cell left the basal layer. We are now testing the hypothesis that overexpression of the transferrin receptor on basal keratinocytes or *de novo* expression of the transferrin receptor on suprabasal keratinocytes can lead to increased accumulation of iron in the epidermis and result in increased iron elimination through desquamation. This would represent dermatoremediation by desquamation.

Restoring genes lost through evolution: During the course of evolution, mutations have inactivated the normal function of several useful genes. For example, the gene for gulonolactone oxidase, the enzyme responsible for vitamin C synthesis (14,15), allows animals other than

primates to make their own vitamin C. Humans and other primates have an inactivating mutation in gulonolactone oxidase and require dietary vitamin C. Correction of the existing inactive gene or introduction of an active gulonolactone oxidase gene into skin could provide a continuous supply of vitamin C to skin, potentially reducing oxidative damage to the skin resulting from carcinogen exposure, UV light or aging (16). Because of the large metabolic capacity of epidermis, restoration of inactive genes in epidermis could have beneficial systemic effects, as well.

In these days of great promise but little practical experience, we can only wonder whether our schemes to engineer better skin will ever match the variety and inventiveness of methods used by parasites to penetrate the skin barrier.

References:

1. Carroll JM, Fenjves ES, Garlick JA, Taichman LB. Keratinocytes as a target for gene therapy. In: Molecular Biology of the Skin, M Darmon and M Blumenberg, eds, Academic Press, Inc, San Diego, CA, 1993.

2. Greenhalgh DA. Rothnagel JA. Roop DR. Epidermis: an attractive target tissue for gene therapy. J Invest Dermatol 103(5 Suppl):63S-69S, 1994

3. Yu WH, Kashani-Sabet M, Liggitt D, Moore D, Heath TD, Debs RJ Topical gene delivery to murine skin. J Invest Dermatol 112:370-375, 1999

4. Barker JNWN, Mitra RS, Griffiths CEM, Dixit VM, Nickoloff BJ. Keratinocytes as initiators of inflammation. Lancet, 337: 211-14, 1991.

5. Kupper TS. The activated keratinocyte: A model for inducible cytokine production cy non-bone marrow-derived cells in cutaneous inflammatory and immune responses. J Invest. Dermatol 94:146S-250S, 1990.

6. Hunyadi J, Simon M, Dobozy A. Immune-associated surface markers of human keratinocytes. Immunol Lett 31:209-216, 1992.

7. Marks C, Marks JD. Phage Libraries -- A new route to clinically useful antibodies. N Engl J med, 335: 730-733, 1996.

8. Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the g or z subunits of the immunoglobulin and T-cell receptors. Proc Nat'l Acad Sci, 90:720-24, 1993.

9. Hwu P, Yang JC, Cowherd R, Treisman J, Shafer GE, Eshhar Z, Rosenberg SA. In vivo antitumor activity of T cells redirected with chimeric antibody T-cell receptor genes. Cancer Res

55: 3369-73, 1995.

10. Schwartz PM, Reuveni H, Milstone LM. Local and systemic implications of thymidine catabolism by human keratinocytes. Annals of NY Acad of Sci 548:115-124, 1989.

11. Schwartz/Taichman

12. Molin L, Wester PO. The estimated daily loss of trace elements from normal skin by desquamation. Scand J Clin Lab Invest 36:679-682, 1976

13. Gatter KC, Brown G, Trowbridge IS, Woolston R-E, Mason DY. Transferrin receptors in human tissues: their distribution and possible clinical relevance. J Clin Pathol 36:539-545, 1983.

14. Nishikimi M, Yagi K. Molecular basis for the deficiency in humans of gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis. Am J Clin Nutr 54:1203S-8S, 1991.

15. Nishikimi M, Fukuyama R, Minoshima S, Shimizu N, Yagi K. Cloning and chromosomal mapping of the human nonfunctional gene for L-gulono-γ-lactone oxidase, the enzyme for L-ascorbic acid biosynthesis missing in man. J Biol Chem, 269: 13685-8, 1994.

16. Darr D, Combs S, Dunston S, Manning T, Pinnell S. Topical vitamin C protects porcine skin from ultraviolet radiation-induced damage. Br. J Dermatol, 127: 247-53, 1992.

Biographical Sketches

Provide the following information for the key personnel listed on page 1 of the Detailed Cost Estimate form for the initial budget period.

NAME	POSITION TITLE
Pauline M. Schwartz, Ph.D.	Prinicipal Investigator

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
Drexel University, Philadelphia, PA	B.S.	1970	Chemistry
University of Michigan. Ann Arbor, MI	M.S.	1971	Med.Chem.
University of Michigan. Ann Arbor, MI	Ph.D.	1975	Med.Chem.

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds two pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED THREE PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INVESTIGATOR.

Professional Experience:

1975 - 1977 Los Angeles County-University of Southern California, Cancer Center
Postdoctoral Research Associate/ Scholar

1977 - 1983 Yale University School of Medicine, Department of Pharmacology
Research Associate (1980-1983),
Postdoctoral Associate/ Fellow (1977-1980)

1983 - 1984 E.I. DuPont DeNemours and Co., Biomedical Products Department
Research Pharmacologist

1984 - Present University of New Haven, Department of Chemistry and Chemical Engineering
Practitioner-in-Residence

1985 - Present Yale University School of Medicine, Department of Dermatology
Research Scientist (1992 - present)
Associate Research Scientist (1985 - 1992)

1985 - Present Veterans' Affairs - Connecticut Healthcare System
Principal Investigator and Pharmacologist (1988 - Present)
Co-Investigator and Health Scientist (1985 - 1988)

Honors and Awards:

Woman of the Year, VA-Connecticut Medical Center, 1996.
Member, Yale Cancer Center, 1996.
Member, Yale Skin Disease Research Center, 1993.
Distinguished Adjunct Professor, University of New Haven, 1992.
VA Merit Award, 1988 - present
NIH Young Investigator Award, 1981-1982.
NIH Postdoctoral Fellowship, 1978-1980.
The Pharmaceutical Manufacturers Association Fellowship, 1971- 1975.
The Wilton R. Earle Award of the American Tissue Culture Association, 1974.
The Alice T. Drexel Scholarship, 1967-1970.

Publications (selected): Pauline M. Schwartz

Schwartz, P.M. and Drach, J.C. Thin-layer chromatography of purine bases, nucleosides and nucleotides. In: Nucleic Acid Chemistry: Improved and New Synthetic Procedures, Methods and Techniques. L.B. Townsend and R.S. Tipson, eds., Wiley and Sons, Part 2, pp 1061, 1978.

Schwartz, P.M. and Handschumacher, R.E. Selective antagonism of 5-fluorouracil cytotoxicity by allopurinol in vitro. Cancer Res., 39:3095, 1979.

Handschumacher, R.E., Schwartz, P.M. and Moyer, J.D. Some consequences of inhibition of the de novo pathway of pyrimidine biosynthesis. In: Antimetabolites in Biochemistry, Biology and Medicine. J. Skoda and P. Langen, eds., Pergamon Press, pp 297, 1979.

Schwartz, P.M., Dunigan, J.M., Marsh, J.C. and Handschumacher, R.E. Allopurinol modification of the toxicity and anti-tumor activity of 5-fluorouracil. Cancer Res., 40:1885, 1980.

Schwartz, P.M., Novack, J.N., Shipman C., Jr. and Drach, J.C. Metabolism of arabinosyladenine in herpes simplex virus-infected and uninfected cells: Correlation with inhibition of DNA synthesis and role in anti-viral selectivity. Biochem. Pharmacol., 33:2431, 1984.

Schwartz, P.M., Turek, P.J., Hyde, C.M., Cadman, E.C. and Handschumacher, R.E. Altered plasma kinetics of 5-fluorouracil at high dosage in rat and man. Cancer Treat. Rept., 69:133, 1985.

Schwartz, P.M., Moir, R.D., Hyde, C.M., Turek, P.J. and Handschumacher, R.E., Role of uridine phosphorylase in the anabolism of 5-fluorouracil. Biochem. Pharmacol., 34:3585, 1985.

Schwartz, P.M., Kugelman, L.C., Coifman, Y., Hough, L.M. and Milstone, L.M. Human keratinocytes catabolize thymidine. J. Invest. Dermatol., 90:8, 1988.

Schwartz, P.M. and Milstone, L.M. Thymidine phosphorylase in human epidermal keratinocytes. Biochem. Pharmacol., 37:353, 1988.

Schwartz, P.M., Reuveni, H. and Milstone, L.M. Local and systemic implications of thymidine catabolism by the human epidermis. Proc. NYAS, 548:115, 1988.

Schwartz, P.M. and Milstone, L.M. Dipyridamole potentiates the growth-inhibiting activity of methotrexate and 5-fluorouracil in human keratinocytes in vitro. J. Invest. Dermatol., 93:523, 1989.

Reuveni, H., Bull, C.O., Landry, M.L., Milstone, L.M. and Schwartz, P.M. Antiviral activity of 5-iodo-2'-deoxyuridine and related drugs in human keratinocytes infected in vitro with herpes simplex virus, type 1. Skin Pharmacol. 4: 291, 1991.

Schwartz, P.M., Barnett, S.K. and Reuveni, H. Thymidine salvage changes with differentiation in human keratinocytes in vitro. J. Invest. Dermatol., 97: 1057, 1991.

Schwartz, P.M., Barnett, S.K., Atillasoy, E.J. and Milstone, L.M. Methotrexate induces differentiation of human keratinocytes. Proc. Natl. Acad. Sci., USA, 89:594, 1992.

Schwartz, P.M., Barnett, S.K. and Milstone, L.M. Keratinocytes differentiate in response to inhibitors of deoxyribonucleotide synthesis. J. Dermatologic Sci. 9: 129, 1995.

Fenjves, E.S., Schwartz, P.M. Blaese, R.M., and Taichman, L.B. Keratinocyte gene therapy for adenosine deaminase deficiency: A model approach for inherited metabolic disorders. Human Gene Therapy, 8: 911-917, 1997.

Schwartz, P.M., Haggerty, J.G. and Cheng, Y-C. β-L-1,3-Dioxolane-cytidine: A novel nucleoside that inhibits proliferation and induces differentiation of keratinocytes in vitro. Skin Pharmacol, 11: 207-213, 1998.

Asgari, M.M., Haggerty, J.G., McNiff, J.M., Milstone, L.M. and Schwartz, P.M. Expression and localization of thymidine phosphorylase/platelet-derived endothelial cell growth factor in skin and epidermal tumors. J Cut. Pathol. 26: 287-294, 1999.

Milstone, L.M. and Schwartz, P.M. Engineering better skin. In: Skin, The Barrier Zone, S. Klaus and J. Hamburger, eds. (in press).

PATENTS:

Milstone, L.M. and Schwartz, P.M. Compositions and Methods for Treating Cutaneous Hyperproliferative Disorders. Issued September 7, 1993. Patent Number: 5,242,921.

Milstone, L.M. and Schwartz, P.M. Method for the Treatment of Hyperproliferative Disorders. Issued July 5, 1994. Patent Number: 5,326,764.

Provisional Patents:

Schwartz, P.M. Epidermal Biosensors: Continuous, Early, Sensitive Monitors for Onset of Specific Diseases.

Schwartz, P.M. An Epidermal Biosensor for Carcinoembryonic Antigen.